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**Gene Structure of the Kiss1 Receptor-2 (*Kiss1r-2*) in the Atlantic Halibut:
Insights into the Evolution and Regulation of *Kiss1r* Genes**

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Running title: Structure of *Kiss1r-2* in *Hippoglossus hippoglossus*

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Abstract

Kisspeptin and its receptor, Kiss1r, play an essential role in the control of the onset of puberty in vertebrates. We characterized the cDNA and genomic DNA encoding Kiss1r in Atlantic halibut (*Hippoglossus hippoglossus*). The 1,146 bp open reading frame predicts a 381 amino acid protein with high homology to the Kiss1r-2 of other teleost fish. Phylogenetic analysis of Kiss1r sequences suggests that the mammalian Kiss1r-1 form arose by way of a gene duplication prior to the emergence of amphibians. Synteny analysis demonstrated the highly conserved nature of the *Kiss1r-2* region in teleosts, suggesting that flanking regulatory sequences are also likely to be conserved. Bioinformatic analysis identified six conserved regions in piscine *Kiss1r-2* upstream sequences, providing potential targets for future in-depth investigation of *Kiss1r-2* regulation. *Kiss1r-2* expression in the brain increased coinciding with the onset of puberty. Expression levels in the gonads were two orders of magnitude lower than those of the brain, a characteristic apparently conserved in other fishes, and expression in gonads was only detected in immature fish.

Keywords: Kisspeptin, *Kiss1r*, Kiss-1 receptor, *gpr54*, puberty, Atlantic halibut.

1. Introduction

Puberty is a cascade of developmental events that culminates in a mature adult reproductive system (Schulz and Goos, 1999). This process is regulated by a series of actions along the hypothalamic-pituitary-gonadal (HPG) axis in mammals (Ojeda, 2006) and fishes (Weltzien et al., 2004; Taranger et al., 2009). The hypothalamic neurons stimulate the production of the gonadotropin-releasing-hormone (GnRH) which, in turn, activates the pulsatile secretion of two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Funes et al., 2003; Gottsch et al., 2004; Messenger et al., 2005). A recently described system consisting of the peptide hormone kisspeptin and its receptor have been demonstrated to be crucial in the initiation of puberty in vertebrates (de Roux et al., 2003; Funes et al., 2003) by regulating the HPG. In mouse (*Mus musculus*) and rat (*Rattus norvegicus*), mutations of the receptor gene, *Kiss1r*, result in hypogonadotrophic hypogonadism, a syndrome characterized by reduced secretion of gonadotropins, and subsequent abnormal pubertal development (de Roux et al., 2003; Seminara et al., 2003). Puberty regulation by the kisspeptin-Kiss1r system appears to be conserved in fish, amphibians and mammals (Roseweir and Mir, 2009).

The kisspeptin receptor (Kiss1r) was first identified in rat as an orphan G-protein coupled receptor (Lee et al., 1999). In 2001, the role of this protein as a receptor for kisspeptin was defined (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). This gene has been subsequently identified in many mammalian species, including human (*Homo sapiens*) (Muir et al., 2001), mouse (Stafford et al., 2002), rhesus macaque (*Macaca mulatta*) (Shahab et al., 2005) and pig (*Sus scrofa*) (Li et al., 2008) among others. Kiss1r contains

seven transmembrane domains (TMDs) that are highly conserved among species, a feature that facilitates the cloning of the *Kiss1r* in additional taxa. This approach has been used to clone *Kiss1r* in lower vertebrates including an amphibian, the bullfrog (*Rana catesbeiana*) (Moon et al., 2008) and several fishes: Nile tilapia (*Oreochromis niloticus*) (Parhar et al., 2004), cobia (*Rachycentron canadum*) (Mohamed et al., 2007), grey mullet (*Mugil cephalus*) (Nocillado et al., 2007), fathead minnow (*Pimephales promelas*) (Filby et al., 2007), zebrafish (*Danio rerio*) (Biran et al., 2008), Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2009) and goldfish (*Carassius auratus*) (Li et al., 2009). Additionally, *Kiss1r* has been identified in the genome sequences of other species including medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), pufferfish (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*), clawed frog (*Xenopus tropicalis*) and the lesser hedgehog (*Echinops telfairi*) (Lee et al., 2009; Mechaly et al., 2009).

Recently, a second form of kisspeptin, Kiss2, which shows amino acid differences in three positions compared to Kiss1, has been identified in non-mammalian vertebrates (Felip et al., 2009; Kitahashi et al., 2009; Lee et al., 2009). As well, multiple copies of *Kiss1r* have been detected in some species: two *Kiss1r* are present in zebrafish, medaka (Lee et al., 2009), goldfish (Li et al., 2009) and platypus (*Ornithorhynchus anatinus*), while three are present in clawed frog (Lee et al., 2009). Amino acid similarity and synteny analyses suggest that two *Kiss1r* lineages (*Kiss1r-1* and *Kiss1r-2*) arose through gene or chromosome duplication with a second gene duplication occurring in the *Kiss1r-1* lineage to produce the amphibian *Kiss1r-1a* and *Kiss1r-1b* genes (Lee et al., 2009). Most fish *Kiss1r* characterized to date fall into the *Kiss1r-2* class (Mechaly et al., 2009). The two paralogous *Kiss1r* genes detected in zebrafish, named *Kiss1ra* and *Kiss1rb*, showed differences in their pattern of gene expression during

maturation. Thus, while *Kiss1ra* expression was higher during puberty and low in mature fish, *Kiss1rb* expression levels were consistent throughout these stages (Biran et al., 2008). Pleuronectiformes, along with the order Tetraodontiformes, constitute a modern and derived group of teleosts. With the limited information available so far, it seems that in these orders the ligand and its receptor, *Kiss1r-1* have been lost during evolution, but this needs confirmation in more species. In the Senegalese sole, only a single *Kiss1r* was identified. However, two different transcript variants were detected (Mechaly et al., 2009). The two variants (named Ss *Kiss1r_v1* and Ss *Kiss1r_v2*) originated from a single gene by alternative splicing that involved retention of the entire intron III. The two isoforms of Senegalese sole also showed differences in their expression pattern: Ss *Kiss1r_v1* had a higher expression than the Ss *Kiss1r_v2* in the brain while the opposite occurred in gonads (Mechaly et al., 2009). The different gene copies and isoforms of *Kiss1r* complicate the understanding of the roles of these genes in controlling the onset puberty in vertebrates. Whether the lack of two different genes in these fish is compensated by the appearance of splice isoforms is still not known.

Human *KISS1R* is expressed in many tissues, including placenta, pituitary, spinal cord pancreas, brain, stomach, intestine, thymus, spleen, lung, testis, kidney and liver (Kotani et al., 2001) suggesting pleiotropic functions for this system. In puberty regulation, the focus has been the brain and the gonads, two organs that play crucial roles in the control of maturation during different stages of development (Nocillado and Elizur, 2008). Expression of *Kiss1r* during development in fishes shows a variety of patterns. For instance, in the brain of Nile tilapia *Kiss1r* expression was associated with three GnRH neuron types (GnRH1, GnRH2 and GnRH3) (Parhar et al., 2004) and showed a significant increase of expression during gonadal maturation (Parhar et al., 2004). A similar pattern was observed in cobia, with an increase of

mRNA levels of *Kiss1r* in the brain during the early stages of puberty (Mohamed et al., 2007). However, *Kiss1r* expression in the brain of the grey mullet was elevated during early puberty but decreased during advanced stages (Nocillado et al., 2007). In the brain of fathead minnow, *Kiss1r* mRNA peaked before the onset of puberty and decreased thereafter (Filby et al., 2007). Finally, in the Senegalese sole, where two splice isoforms were detected, the expression in the brain was higher in pubertal fish than mature fish, and Ss *Kiss1r v1* expression was higher than *Kiss1r v2* in both stages (Mechaly, et al., 2009). This variation in expression pattern between species may be due to species differences or, alternatively, to the presence of different genes or splice variants of the same gene (Mechaly et al., 2009). Nevertheless, this is another aspect of kisspeptin regulation that needs to be clarified.

The Atlantic halibut, *Hippoglossus hippoglossus* (Hh), belongs to the order Pleuronectiformes and thus presumably has only one *Kiss1r* gene, but whether it has a splice mechanism or not it not known. Atlantic halibut is the largest and longest-lived flatfish, capable of growing to sizes of over 2.2 m and of living for more than 50 years (Haug, 1990). In addition, Atlantic halibut are slow to mature, requiring 5–7 years. Halibut females generally grow more quickly than males and attain a larger ultimate size (Douglas et al., 2006). Early maturation and reduced growth rates of males are one of the major problems for commercial halibut aquaculture (Norberg et al., 2001). Thus, understanding the control of the initiation of puberty in this species not only has basic interest in knowing about the kisspeptin-*Kiss1r* system in a flatfish, but may also have potentially significant implications for the halibut aquaculture industry. As a first step in better understanding the onset of puberty in the Atlantic halibut, *Kiss1r* was cloned and its expression analyzed in a variety of tissues at multiple stages of development.

2. Experimental Procedures

2.1. Fish and sampling

Juveniles and adults of Atlantic halibut were obtained from Scotian Halibut Ltd. (Woods Harbour, NS). Fish were kept in tanks appropriate for their developmental stage under natural conditions of photoperiod and temperature and were fed twice a day with a commercial diet. These conditions allow Atlantic halibut to mature in captivity. Animals were treated according to the guidelines of the Canadian Council on Animal Care (Demers, 2006) and protocols were approved prior to experimentation by all relevant authorities. Four different year classes (one, three, five and seven year-old) were sampled. Fish were anesthetized with an overdose of MS-222 (Sigma-Aldrich, St. Louis, MO) and sacrificed by decapitation. Tissues were quickly removed under RNase-free conditions, flash frozen in liquid nitrogen and stored at -80°C until used. Gonads (testis and ovaries) were fixed in 4% paraformaldehyde for further histological examination. Fin clips were taken and stored in 95% ethanol for DNA extraction. Biometric information including standard length (cm) and weight (g) were obtained for all fishes analyzed.

2.2. Histological analyses

After fixation in 4% paraformaldehyde for approximately 24 hrs at room temperature, gonads were washed for an additional 24 h in phosphate buffer (PB) (pH 7.4), dehydrated in an ascending series of ethanol concentrations, cleared in xylene (Fisher Scientific, WA, USA) and embedded in Paraplast (Fisher Scientific, Pittsburgh, PA). Sections (7 µm thick) were cut using a rotary microtome (Microm HM-355), float mounted on Colorfrost/Plus slides (Fisher

Scientific), warmed on a slide warmer for 1 h and stained with Instant Haematoxylin and Eosin (H-E) (Sigma). Stained sections were mounted in Permount (Fisher Scientific) under coverslips, examined on a DMRE Leica light microscope and photographed using Compix imaging software. Stages of gametogenesis were determined according to the different germ cell types present as previously described (Hendry et al., 2002).

2.3. Partial cloning of Atlantic halibut *Kiss1r* gene

Total RNA was isolated from frozen tissue with TRIZOL Reagent (Invitrogen), its quality checked in a 1.5% agarose gel stained with SYBR safe (Syber Safe™, Invitrogen) and its quantity measured in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc, Wilmington, DE, USA). All RNAs were treated with DnaseI (Ambion) to avoid genomic contamination of the RNA samples. cDNA synthesis was performed following the manufacturer's instructions using 1 µg of RNA with Superscript III Reverse Transcriptase and random hexamer primers (Invitrogen) in a 20 µl reaction volume.

A partial cDNA sequence of Hh *Kiss1r* was first isolated by reverse transcription PCR (RT-PCR) using degenerate oligonucleotides (Hh_Kiss1r-F3 and Hh_Kiss1r-R3) (Table 1) that were designed on the basis of the previously published sequences of Nile tilapia (**AB162143**) (Parhar et al., 2004), cobia (**DQ790001**) (Mohamed et al., 2007), Senegalese sole (**EU136710**) (Mechaly et al., 2009), Atlantic croaker (*Micropogonias undulates*) (**DQ347412**) and grey mullet (**DQ683737**) (Nocillado et al., 2007). PCR reactions were carried out using 1 µl of cDNA in a final volume of 25 µl containing 2.5 µl of 10X PCR reaction buffer (0.75 mM MgCl₂), 0.2 mM of each dATP, dCTP, dGTP, dTTP, 50 pmol of each primer and 0.2 µl of rTaq DNA polymerase (G.E. Healthcare). Cycling parameters

consisted in an initial heat denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, and finished with a final extension step at 72°C for 5 min. Bands of the expected size were purified (Montage PCR, Millipore), ligated into pCR2.1-TOPO vector (Invitrogen), using the TOPO TA cloning method and transformed into *E. coli* competent cells following the manufacturer's instructions. White colonies were selected from X-Gal/IPTG ampicillin agar plates and grown in LB/ampicillin liquid media. Plasmid DNAs were purified with QIAprep Spin Miniprep (QIAGEN) and used as templates in sequencing reaction. The plasmid DNA was purified and sequenced with universal primers using an ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). To avoid sequencing errors, ten different plasmids DNAs were sequenced in both directions. The resulting DNA sequences were compared by BLAST analysis to known piscine *Kiss1r* cDNA sequences available in GenBank.

2.4. BAC library screening

An Atlantic halibut BAC library composed of 35,328 clones with an average insert size of 100 kb was constructed by Lucigen Corp. (Middleton, WI). Based on the partial Hh *Kiss1r* sequence, primers for an overgo probe (Overgo-Kiss1r-F1 and Overgo-Kiss1r-R1) were designed using Soop (system for optimized overgo picking software; Thomas et al., 2002) for library screening. Overgo probes were labeled according to Ross et al. (1999). Briefly, annealing of the overgo oligo pairs (10 pmol each) was done in 4.5 µl of water at 80°C for 5 min followed by 37°C for 10 min and then stored on ice. Labeling was carried out by adding 2.0 µl 5X OLB –{A, C}, 0.5 µl of 2 mg/ml acetylated BSA (New England Biolabs, Ipswich, MA), 0.5 µl each of [α -³²P] dATP and [α -³²P] dCTP (10 µCi/µl, ~3000Ci/mmol, Perkin Elmer, Shelton, Connecticut) and 1 µl (2U/µl) of Klenow enzyme (New England

Biolabs, Ipswich, MA). For ease of image alignment, an overgo probe from *E. coli* was also labeled. The reactions were incubated at room temperature for 1 h. Fifteen minutes before the end of the incubation 1 μ l of a cold chase (200 μ M each of dATP and dCTP) was added. Unincorporated dNTP's were removed using NICK Sephadex G50 columns (GE Healthcare Baie d'Urfe, Quebec). Filters were pre-hybridized in roller bottles in a hybridization oven using Church's hybridization buffer (Ross et al., 1999) containing 0.5 ml sonicated salmon sperm DNA (10 mg/ml) (Ambion, Austin, Texas) that had been heated to 100°C for 5 min immediately before use. Pre-hybridization was carried out for 3 h at 55°C. The pre-hybridization solution was then replaced with 25 ml of fresh Church's buffer containing salmon sperm DNA to which was added the labeled denatured (95°C for 10 min) *Kiss1r* and *E. coli* (2% of the *Kiss1r* volume) overgo probes. Hybridization was carried out for 72 h at 55°C. Following hybridization the filters were rinsed once with 4X SSC containing 0.1% SDS at room temperature and then extensively washed at 55°C for 30 min: 2 X 1 liter each of 2X SSC/0.1% SDS, 1X SSC/0.1% SDS and 0.5X SSC/0.1% SDS. The filters were then exposed to Bio MS film (Kodak) with Biomax MS intensifying screens (Kodak) at -80°C for 24-48 h.

2.5. Genomic structure of Hh *Kiss1r* gene

Positive clones were isolated, tested by PCR and purified using a Qiagen Large Construct Kit (Qiagen). The BAC DNAs were sequenced on a Roche GS-FLX by the McGill University and Genome Quebec Innovation Centre. The contigs returned from the GS-FLX sequencing had a gap in intron I of the Hh *Kiss1r* gene. To obtain the complete gene sequence, genomic DNA was amplified with primers located in intron I (Intron I-F1 and Intron I-R1) (Table 1). PCR reactions were carried out in a final volume of 25 μ l containing 2.5 μ l of 10x PCR reaction buffer with 0.75 mM of MgCl₂, 0.2 mM of each dATP, dCTP,

dGTP, dTTP, 50 pmol of each primer and 0.2 µl of rTaq DNA polymerase (G.E. Healthcare). PCR was carried out with an initial heat denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 92°C, 30 s at 55°C and 30 s at 72°C, and finished with the final extension step at 72°C for 5 min. PCR products were analyzed on 1.5% agarose gels and bands of the expected size were excised and purified by gel extraction (Qiaquick-Qiagen). The PCR products were directly sequenced by Genewiz Inc. (South Plainfield, NJ). Partial sequences were assembled and edited using Bioedit software version 7.0.5.3 (Hall, 1999).

2.6. DNA sequence and phylogenetic analysis

The location of the putative signal peptide cleavage site of halibut Kiss1r was predicted using the SignalP server of the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/SignalP/>). The seven TMDs helices were predicted by the HMMTOP Server (Tusnády and Simon, 2001). Upstream sequences of fish *Kiss1r-2* were analyzed with Meme (Bailey and Elkan, 1994) and conserved regions were compared to the Transfac database (Wingender et al., 1996) with Tomtom (Gupta et al., 2007). Multiple alignments and phylogenetic analysis was performed using the amino acid sequences of the seven TMDs of Kiss1r available from GenBank and Ensembl. The sequence sources and GenBank accession numbers were as follow: dog (*Canis lupus familiaris*) (**XM 850105**), sheep (*Ovis aries*) (**ABY61032**), rhesus macaque (**XP 001117198**), mouse (**NP 444474**), rat (**AAD19664**), pig (**ABE73453**), human (**AAK83235**), chimpanzee (*Pan troglodytes*) (**XR 020820**) opossum (*Monodelphis domestica*) (**XM 001374715**), platypus (platypus-1 **Ultra 266:390150;395924**; platypus-1B, **Contig 15629:1100:6789**), clawed frog (clawed frog-1A, **EU85678**; clawed frog-1B **EU853679**; clawed frog-2, **EU853680**), medaka (medaka-1, **Chromosome 9:4480521:4500733**; medaka-2, **Chromosome**

17:29839761:29855706); zebrafish (zebrafish-A, **ABV44612**; zebrafish-B, **ABV44613**); goldfish (goldfish-A, **ACK77792**; goldfish-B, **ACK77793**); bullfrog (**ACD44939**), fathead minnow (**ABV45419**), Nile tilapia (**BAD34454**), Senegalese sole (**ABW96362**), grey mullet (**ABG76790**), cobia (**ABG82165**), Atlantic croaker (**ABC75101**) and Atlantic halibut (**GQ330487**). Additionally, sequences for several species were retrieved from genome sequences (<http://www.ensembl.org>): three-spined stickleback, pufferfish, fugu, lesser hedgehog (Mechaly et al., 2009) and Anole lizard (*Anolis carolinensis*), pika (*Ochotona princeps*), guinea pig (*Cavia porcellus*), megabat (*Pteropus vampyrus*), microbat (*Myotis lucifugus*), kangaroo rat (*Dipodomys ordii*), gorilla (*Gorilla gorilla*), bushbaby (*Otolemur garnettii*), dolphin (*Tursiops truncatus*), orangutan (*Pongo pygmaeus*) and mouse lemur (*Microcebus murinus*). Sequences were aligned using Clustal W (Thompson et al., 1994) and edited in Bioedit (Hall, 1999). Sequence alignment was based on the TMDs because adjacent regions were difficult to align and therefore to assign homology among the amino acid residues. A phylogenetic tree was constructed using the neighbor-joining method (Saitou et al., 1987) using amino acid Poisson distance. Evaluation of statistical confidence in nodes was based on 500 nonparametric bootstrap replicates (Felsenstein et al., 1985). All phylogenetic analysis were conducted on MEGA version 4 (Tamura et al., 2007)

2.7. Tissue-specific expression of Hh *Kiss1r*

Total RNA isolation and cDNA synthesis from brain, ovary, testis, liver, muscle, stomach, heart, intestine and kidney, from three and seven year old males and females were carried out following the protocol described in section 2.3. The resulting cDNAs were used to amplify Hh *Kiss1r* by RT-PCR using the RT Hh Kiss1r-F2 and -R2 primers (Table 1). The PCR cycling conditions for Hh *Kiss1r* were: 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at

55°C, 30 s at 72°C and a final extension of 5 min at 72°C. Amplification of *β -actin* RNA was done as a control using the RT Hh β actin-F1 and -R1 primers (Table 1) under the same conditions. A no-template control (NTC) was included to ensure that contamination of reagents had not occurred. PCR products were resolved on a 1% agarose gel stained with ethidium bromide.

2.8. Microsatellite analysis

For immature fish with undeveloped gonads, sex was determined by genotyping with five microsatellite markers (Hhi4IMB, Hhi39IMB, Hhi52IMB, Hhi62IMB, Hhi68IMB; Reid et al., 2007) to establish their pedigree and sex was subsequently inferred from the inheritance of paternal alleles (D. Reid, M. Reith, unpublished). In this case, genomic DNA was extracted from fin clips (8–10 mg) using the Puregene DNA purification/extraction kit (Gentra Systems, USA) as per the solid-tissue extraction method. The standard PCR cocktail included: 10X high fidelity buffer (20mM Tris-HCl, 50 mM KCl, 1.5mM MgCl₂) (Larova GmbH, Germany), 200 μ M of each dNTP; 0.4 to 0.8 pmol each of forward and reverse primer, 0.25 units of high fidelity Taq DNA polymerase (Larova GmbH, Germany) and 0.05 μ l of BSA. The PCR reactions contained 9.5 μ l (8.4 μ l PCR cocktail and 1.1 μ l of 50 ng/ μ l DNA) reactions in a 96 well polypropylene microplate and were run on a Techne TC-412 (Techne, USA). PCR conditions were an initial denaturing of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C (for markers 3 and 5) or 58°C (for markers 1, 2 and 4) for 30 s and 72°C for 45 s and finally 5 min. at 72°C. Markers 1, 2 and 4 were run in a single reaction and markers 3 and 5 were run in a second reaction. For each sample, 1.5 μ l of the first PCR and 2.5 μ l of the second PCR were combined with 35 μ l CEQ sample loading solution and 0.5 μ l of internal DNA size standard (CEQ) into a CEQ sample plate. Pedigree analysis was

performed using PROBMAX software (Danzmann 1997) (<http://www.uoguelph.ca/~rdanzman/software/PROBMAX/>) using previously collected genotypes from potential parents.

2.9. Determination of Hh *Kiss1r* mRNA levels in brain and gonads

The expression pattern of Hh *Kiss1r* was analyzed in whole brain and ovaries and testis from four different year-classes (1, 3, 5 and 7 years old) by real-time quantitative PCR (qPCR). RNA was isolated as indicated above and it was further purified with a RNA cleanup column (Qiaprep, Qiagen) and a second DNase treatment, in which 20 µg of purified RNA were treated with 2 µl of DnaseI (Ambion) and 1 µl of DnaseI Reaction Buffer in a final volume of 25 µl. Aliquots of the RNA were analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies) to check the quality of the samples. The Hh *Kiss1r* primers used for qPCR were located in exons 3 and 4 and designed to span intron III (RT Hh Kiss1r-F2 and RT Hh Kiss1r-R2) (Table 1).

The qPCR amplification reaction mixture contained 2 µl of diluted cDNA (1:3) (freshly synthesized from 1 µg of RNA), 4 µM of each primer, and 10 µl of SYBR Green PCR Master Mix (Roche) in a final volume of 20 µl. Thermal cycling conditions comprised heating to 95°C for 10 min to activate the FastStart Taq DNA Polymerase (Qbiogene, Inc), 40 cycles at 95°C for 10 s and 60°C for 30 s. At the end of the PCR cycles, the qPCR products were immediately analyzed using a dissociation curve step to confirm that only a single PCR product was amplified. No-template control reactions for every primer pair were also included on each reaction plate to check for external DNA contamination. The amplification efficiency (E) of each primer set/gene target was determined in validation assays containing serially diluted cDNA templates (1:3, 1:10, 1:30, 1:100, 1:300 and 1:1000). Efficiency was estimated

as $E = 10 (-1/\text{slope})$. The amplification efficiencies were 1.959 and 1.989 for the Hh *Kiss1r* and *β -actin*, respectively. The slopes of the curve were -3.424 and -3.349 and the melting temperatures (T_M) were 86.4 and 82, respectively. A single peak was obtained from each gene during melt curve analysis, demonstrating specificity of the primers. PCR products were resolved on 1% agarose gel stained with ethidium bromide to verify the expected size of the band.

Data were analyzed using the comparative Ct method (Bustin, 2000) (also known as the DDCT method) to calculate relative changes in gene expression using the Light Cycler 480 Software (release 1.5.0, Roche). Fold change (or relative quantification, RQ) was calculated from the DDCT. The LightCycler Relative Quantification Software (Roche) was used for determining calibrator-normalized target/reference ratios with automated efficiency-corrected quantification according to the recommendations of the manufacturer. Fold change was normalized to the endogenous reference gene *β -actin*. Determinations were carried out in technical triplicates for the Hh *Kiss1r* and *β -actin* genes. The RQ values for each sample were averaged and the standard error was calculated, yielding the average fold change of the target gene.

2.10. Statistical analyses

The results are presented as mean $\pm$ standard error of the mean (S.E.M). Statistical analyses were performed using the SPSS v16 Software. The significance of pairwise comparisons of the mRNA levels of Hh *Kiss1r* in brain of males and females was determined using a Student's t-test. Expression levels of Hh *Kiss1r* in brain during maturation were analyzed by a one-way ANOVA. Differences were accepted as significant when $P < 0.05$.

3. Results

3.1. Cloning and sequencing of the full-length Hh *Kiss1r-2* gene

Using degenerate primers located within exon 5, a 202 bp fragment of Hh *Kiss1r-2* was obtained by PCR. The full-length Hh *Kiss1r-2* gene was obtained by screening a BAC library with an overgo probe based on the initial Hh *Kiss1r-2* partial sequence. Two positive clones, both containing the complete Hh *Kiss1r-2*, were isolated, purified and sequenced. Sequence assembly resulted in a small number (6-12) of contigs for each BAC clone, with Hh *Kiss1r-2* split between two contigs in both cases. One contig contained upstream sequences, exon I and the beginning of intron I, while the second contig contained the end of intron I, the remaining coding region and downstream sequences. PCR reactions using primers flanking the gap between the *Kiss1r-2* contigs (Intron I-F1 and R1) were used to complete the sequence of intron I. The complete Hh *Kiss1r-2* sequence was assembled and deposited in GenBank (accession number **GQ330487**).

3.2. Characterization of the Hh *Kiss1r-2*

The coding region of the Hh *Kiss1r-2* spans 9,338 bp, which includes 1,146 bp for the five exons of the open reading frame and 8,192 bp for the four introns (2,425, 5,308, 94 and 365 bp for introns I, II, III and IV, respectively). A putative poly-A signal, AAAAAACAAAAA, was found in the presumptive 3' UTR. Alignment of the Hh *Kiss1r-2* sequence with those of other teleost fish demonstrated a high degree of nucleotide identity (> 86%). The sizes of the five exons of the *Kiss1r-2* gene from the two pleuronectiformes fishes, Atlantic halibut and Senegalese sole were very similar in length, whereas the sizes of the introns varied (Table 2). Translation of the complete Hh *Kiss1r-2* coding region predicts a

381 amino acid protein that has a high degree of sequence similarity to Kiss1r proteins from other fish. As in other Kiss1r, seven transmembrane domains were detected (Figure 1 and Figure 2), as well as a 47 amino acid signal peptide (Figure 2).

3.3. Synteny analysis of Hh *Kiss1r-2*

The larger of the two BAC clones encoding Hh *Kiss1r-2* contained sequences encoding several flanking genes, including a partial *atp6v0b* (encoding V-type proton ATPase 21 kDa proteolipid subunit), three microRNA genes (*miR-23a*, *miR-27a* and *miR-24*), *rabL5* (encoding rab-like protein 5) and a partial *odf2L* (encoding sperm tail outer dense fiber protein 2-like). The organization of genes around the Hh *Kiss1r-2* locus was compared to that of other fish *Kiss1r-2* with available genome sequences (Figure 3). Interestingly, the *atp6v0b~Kiss1r-2~miR-23a~miR-27a~miR-24* gene organization is conserved in all six species, with *b4galt2* located upstream of *atp6v0b* in the species with full genome sequences. Downstream of the microRNA genes, *rabL5~odf2L* is conserved in medaka, three-spined stickleback and Atlantic halibut, while *atp5L* is present in both pufferfish and fugu. The conservation of flanking genes among fish *Kiss1r-2* clearly supports a common ancestry, with subsequent taxa-specific rearrangements downstream of micro RNA genes.

3.4. Phylogenetic analysis of Kiss1r

The high conservation in the TMDs, together with new information for *Kiss1r* from a large number of species, facilitated the alignment of 42 Kiss1r amino acid sequences. Of the 36 species analyzed, five of them contained more than one paralogous gene, with the clawed frog being the only species with three genes and medaka, zebrafish, goldfish and platypus with two genes. The phylogenetic analysis (Figure 4A) clustered all Kiss1r sequences from

placental mammals in a single group. Monotreme (platypus) and marsupial (opossum) mammalian *Kiss1r-1* sequences are represented by branches at the base of this cluster. Similarly, a strongly supported (97%) cluster groups all fish *Kiss1r* sequences along with those from some reptiles and amphibia. Following the scheme of Lee et al. (2009), the cluster containing all mammalian plus the clawed frog-1A sequences is designated *Kiss1r-1*, while the cluster containing all piscine sequences plus sequences from lizard and some amphibia is designated *Kiss1r-2*. In turn, the *Kiss1r-2* cluster could be subdivided into two. One, named *Kiss1r-2A* contained all piscine species that have only one *Kiss1r* gene plus two species of amphibia, and the other, named *Kiss1r-2B*, containing one reptile (anole lizard), amphibian (clawed frog) and those fish with a second *Kiss1r* gene (which were originally named medaka-1, goldfish-B, zebrafish-B). *Kiss1r-2B* sequences were intermediate between the *Kiss1r-2A* sequences and mammalian sequences (*Kiss1r-1*).

3.5 Analysis of the putative Hh *Kiss1r-2* promoter region

The 980 bp upstream sequence from Hh *Kiss1r-2* along with 1500 bp of sequence upstream of the ATG start from the zebrafish, medaka, three-spined stickleback, fugu and pufferfish *Kiss1r-2* were analyzed for conserved sequence regions using Meme (Bailey and Elkan, 1994). Six conserved regions were identified on the coding strand that ranged in size from 11 to 29 bp (Figure 5). All conserved regions fall within an ~1100 bp region upstream of the *Kiss1r-2* ATG. Two of these regions showed considerable similarity to the binding sites for the transcription factors MIF-1 (black boxes in Figure 5) and estrogen receptor (grey boxes). Notable are the conserved location of the estrogen receptor-like sequences near -100 in five of six species and the conserved, adjacent arrangement of the regions represented by the black and white boxes in four of the species.

3.6. Determination of the sex and development stages of Atlantic halibut

Sex was determined and fish were classified to their stage of maturation based on the different types of germinal cells present in gonads (Weltzien et al., 2002; Hendry et al., 2002). Sex in several immature fish with undeveloped gonads was determined by pedigree analysis and the inheritance of a sex-specific microsatellite marker (D. Reid, M. Reith, unpublished). The histological analysis (Figure 6) confirmed that all fish from the one-year class were immature, with spermatogonia present in the males and primordial oocytes in females. The three-year-old fish were entering the early stages of puberty, as determined by the presence of spermatocytes and previtellogenic oocytes. The five-year-old fish were in middle of sexual development, showing spermatids in males, and vitellogenic oocytes in females. Finally, the seven-year class fish were sexually mature with late spermatogenesis evident in males and late vitellogenesis in females.

3.7. Tissue distribution of Hh Kiss1r-2 in early puberty and adult fish

The expression of Hh Kiss1r-2 was analyzed by RT-PCR in different tissues from fish from two year-classes (three- and seven-year-old fish), which corresponded to two different maturation stages, early puberty and mature, respectively. Hh Kiss1r-2 was most highly expressed in the brain in all four combinations of sex and year class (Figure 7). In the three-year-old fish, Hh Kiss1r-2 expression was detectable in most tissues at low levels, with intestine and stomach showing intermediate expression levels in the male and stomach and kidney having intermediate levels in the female. In the seven-year-old fish, Hh Kiss1r-2 expression could only be detected in the brain and stomach in both sexes.

3.8. Expression of Hh *Kiss1r-2* in brain and gonads during development

Real time PCR (qPCR) was used to quantify the expression of Hh *Kiss1r-2* in brain and gonads from the different year classes. For all four year-classes, *Hh Kiss1r-2* expression in the brain was not significantly different between the two sexes ($P = 0.981$, $P = 0.719$, $P = 0.170$, and $P = 0.555$), and thus values were pooled together. One-way ANOVA showed a significant increase in the expression of Hh *Kiss1r-2* in the brain (Figure 8A) coinciding with the onset of puberty (three-year-old class) in comparison with the rest of the year-classes ($P < 0.05$). On the other hand, in gonads (Figure 8B), Hh *Kiss1r-2* expression was only detected in individuals at an extremely low level compared to the brain (>100-fold lower expression) and only in fish from the one-year-old class. In this class, no differences were detected between sexes ($P = 0.945$). No expression was detected in the rest of the year classes.

4. Discussion

Kiss1r is a G-protein-coupled receptor highly expressed in the brain (Kuohung and Kaiser 2007) and found to be associated with GnRH neurons in different animals, including rat, mice and Nile tilapia (Irwing et al., 2004; Messenger et al., 2005; Parhar et al., 2004). Kiss1r has been linked to the regulation of the initiation of puberty in vertebrates (Smith et al., 2006; Tena-Sempere et al., 2006), although it may have additional physiological functions (Makri et al., 2008; Roa et al., 2008).

The analysis of the genomic structure of the Hh *Kiss1r-2* gene revealed the presence of five exons and four introns, a gene organization that is conserved from teleost fish to amphibians and mammals (van Aerle et al., 2008). Comparison of the coding sequence with

other fish revealed a high degree (>90%) of homology. Comparison to the gene organization in a closely related species, the Senegalese sole (Mechaly et al., 2009), revealed substantial length differences in introns II and IV. Interestingly, intron III of Atlantic halibut and Senegalese sole exhibited a considerable degree of sequence conservation (50% identity) and a similar length, 94 and 81 bp, respectively. In Senegalese sole, intron III missplicing results in a second isoform with a different expression pattern than the full-length form. Similar to the Senegalese sole, the Hh *Kiss1r-2* intron III has a branch point with low sequence conservation compared to the consensus sequence. Despite numerous attempts, alternative *Kiss1r* isoforms could not be detected in a variety of Atlantic halibut tissues. However, since not all tissue-developmental stages combinations could be tested, alternative splicing of Hh *Kiss1r-2* remains a possibility. Alternative splicing of *Kiss1r* has also been detected at intron I in sheep (Genbank accessions [EU272411](#) and [EU272412](#)) and with the deletion of the entire TMD5 in mouse ([EU532436](#) and [EU532437](#)), both of which result in proteins with altered TMDs. Alternative splicing may provide an additional mechanism for regulating the expression of Kiss1r or for directing it to an alternative subcellular location.

An exhaustive phylogenetic analysis of Kiss1r provided insights into its evolutionary history. The phylogenetic relationships described here (Figure 4) are in general in agreement with the evolutionary tree constructed in our previous study (Mechaly et al., 2009), although a close inspection of the newer tree, which contains more species (36 vs 20 in Mechaly et al., 2009) reveals some unexpected relationships and suggests that gene duplications have occurred in this lineage. Placental mammalian Kiss1r sequences cluster tightly together (Kiss1r-1 cluster) with the marsupial and monotreme sequences branching from the base of this cluster. The observation that the platypus-1 and clawed frog-1A sequences share a well-

supported common branch suggests that the clawed frog-1A *Kiss1r* shares a common ancestor with the mammalian *Kiss1r*. Most of the fish sequences cluster in a tight group at the end of the tree while the medaka-1, zebrafish-B and goldfish-B are located on a separate branch. Reptile and amphibian sequences cluster between these branches and distance analysis suggests that the clawed frog-2 and bullfrog sequences are most closely related to the main fish group (*Kiss1r*-2A group), while the clawed frog-1B and anole lizard sequences group with the three outlying medaka-1, zebrafish-B and goldfish-B sequences (*Kiss1r*-2B group). Thus, *Kiss1r*-2B do not cluster with the *Kiss1r*-1 of mammals, as surmised earlier (Biran et al., 2008; Li et al., 2009), but are an additional, non-mammalian form of *Kiss1r*. Our interpretation of *Kiss1r* evolution (Figure 4B) is that an ancestral gene duplication occurred prior to the emergence of fish, leading to the 2A and 2B groups, and that an additional duplication of the 2B gene, prior to the emergence of amphibians, resulted in the ancestor of clawed frog-1A. This latter gene appears to be the only *Kiss1r* version retained in higher vertebrates, assuming it is present in reptiles. A duplication of *Kiss1r-1* appears to have occurred within the monotremes. Thus, the evolutionary relationships within the fish *Kiss1r*-2A group and within the placental mammalian *Kiss1r*-1 group does not reflect the expected organismal evolutionary relationships but rather the evolution of this gene lineage. In this regard, it would be interesting to know the structure and sequence of the *Kiss1r-1* gene(s) in other vertebrates, including Sarcopterygian fishes. Also, as of today, there are still no avian *Kiss1r* sequences that can be identified in public databases, despite several attempts.

Among fish, many species appear to have lost *Kiss1r-2B*. Genome sequences from fugu, pufferfish and three-spined stickleback demonstrate the presence of only the 2A form of *Kiss1r-2*. Extensive searches with multiple degenerate primers have failed to detect a second

Kiss1r-2 in Senegalese sole (Mechaly et al., 2009) and Atlantic halibut (this study). These results suggest that Kiss1r-2B could be lost in some of the most derived teleost orders, including the Pleuronectiformes and Tetraodontiformes, as well as some other Acanthopterygian fish. It is not yet clear whether the loss of Kiss1r-2B was a single event in the evolution of fish or has independently occurred multiple times.

Recent studies have shown that Kiss1 also underwent a duplication in some fishes (Felip et al., 2009), a situation similar to the one described for the Kiss1r gene, whereas in mammals, with the exception of the platypus, only one Kiss1 gene is observed. Interestingly, the three teleost species in which two Kiss1 genes have been reported so far, i.e., zebrafish (Kitahashi et al., 2008), medaka (Kanda et al., 2009) and goldfish (Yang et al., 2009), also have two Kiss1r genes. To the best of our knowledge, there are no species with only one Kiss1 gene and two Kiss1r genes or vice versa.

This study also provides the first synteny analysis of the Kiss1r-2 region. Results clearly support a common ancestry of fish Kiss1r-2 with subsequent rearrangement of the genes downstream of the three miRNA genes showing taxa-specific organization. The highly conserved nature of the Kiss1r-2 region suggests that the flanking regulatory sequences are also likely to be conserved. As a first attempt at defining potential transcription factor binding sites, bioinformatic analysis has identified six conserved regions in fish Kiss1r-2 upstream sequences, two of which show some similarity to known transcription factor binding sites. We are hesitant to suggest that these transcription factors bind to these sites and regulate Kiss1r-2 without further experimental analysis. However, they provide potential targets for future in-depth investigation of Kiss1r-2 regulation in different species.

Analysis by qPCR of *Kiss1r* expression in different developmental stages revealed no major differences in expression between male and female Atlantic halibut. A similar absence of sexual dimorphism in the expression of *Kiss1r* was detected in the brain of fathead minnow (Filby et al., 2008). The Hh *Kiss1r-2* expression level in the brain was significantly higher in early puberty stages than the other stages investigated. A similar pattern of expression throughout development was observed in some teleost fish such as the fathead minnow (Filby et al., 2008) and for the zebrafish-A gene (Biran et al., 2008). However, in other species such as Nile tilapia and cobia, *Kiss1r* expression continues to increase as puberty advances (Parhar et al., 2004; Mohamed et al., 2006).

The expression of Hh *Kiss1r-2* in gonads of 1-year-old halibut was 400-fold lower than brain, which is in the same order of magnitude as the 100-fold difference observed in Nile tilapia (Martínez-Chávez et al., 2008), but higher than the 14-fold difference observed in fathead minnow (Filby et al., 2008) or the 10-fold difference observed in mature female grey mullet (Nocillado et al., 2006). Thus, it seems that regardless of species, the relationship between *Kiss1r-2* expression in the brain and the gonads is in the range of 1–2 orders of magnitude. In the 3, 5 and 7 year classes, Ct values in gonads were too high to accurately determine Hh *Kiss1r-2* expression levels, while the expression levels in ovaries and testis in the 1 year-old-class were not statistically different. Similar results were observed in studies carried out in rats, where the ovarian expression of *Kiss1r* did not fluctuate during ovulation (Castellano et al., 2006). However, in the Senegalese sole, the *Kiss1r v1* isoform showed higher expression in the testis than the ovaries (Mechaly et al., 2009).

In aquaculture, precocious sexual maturation, usually of males, has significant negative economic impact since early maturing fish are smaller and may have diminished flesh quality. Understanding the hormonal signals of puberty, their detection and the genes they regulate should provide tools to monitor the sexual maturation of fish at a molecular level and eventually develop management practices to avoid triggering this process. The isolation of Hh Kiss1r-2 and analysis of its gene expression will aid in understanding puberty in this species and provide insights into the evolution and regulation of this developmentally important gene.

In conclusion, in this study we have described the genomic organization of Atlantic halibut Kiss1r-2 gene. We identified only one Kiss1r gene, Hh Kiss1r-2, and proposed that the flatfish, as well as several other orders of fish, have lost the second gene during the evolution of teleost fish. Further, new phylogenetic relationships were proposed based on the analysis of all available Kiss1r sequences. Synteny analysis demonstrates a conserved gene order around the Kiss1r-2 locus in many teleosts and suggests the likelihood of conservation of regulatory regions. Furthermore, the concordant expression patterns of Senegalese sole Kiss1r v1 and Atlantic halibut Kiss1r-2, which show higher expression in brain, demonstrates the importance of the kisspeptin-Kiss1r system in the brain, compared to the gonads, during early puberty.

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Table 1. Degenerate and specific primers used in the Hh *Kiss1r-2* cloning, screening, Intron I isolation, qPCR and RT-PCR

Primers	Primer sequence (5'→3')	Amplicon size (bp)	Usage
Hh_kiss1r-F3	ATCAAGACDTGGGCCAACTGC	202	Partial cloning
Hh_Kiss1r-R3	ATTGTTGCCTTCCTCYGCNGC		Partial cloning
Overgo-Kiss1r-F1	GAGCCAGCTTCCAAAAGTCCTTCA	40	BAC screening
Overgo-Kiss1r-R1	AAGGGGAAGGTTTTCTGAAGGAC		BAC screening
Intron I-F1	GCAAATGAATGTGCCAGTGT	~800	Intron I cloning
Intron I-R1	TGCTGTAGGTGCTTTGAGGA		Intron I cloning
RT Hh Kiss1r-F2	GGTCCAGGCCACTTGTATCA	116	qPCR and RT-PCR
RT Hh Kiss1r-R2	TGCTGACGATCATGGCTACT		qPCR and RT-PCR
RT Hh β actin-F1	AAGAAGAAAGAAAAGACGGAGGA	84	qPCR and RT-PCR
RT Hh β actin-R1	AAGATTTGCCAGAGATGTTTCC		qPCR and RT-PCR

Notes: Y =C or T; D = A, G or T; N =A, C, G or T

Table 2. Intron and exon size in Atlantic halibut *Kiss1r-2* gene (Hh *Kiss1r-2*) and Senegalese sole (Ss *Kiss1r*)

	Hh <i>Kiss1r-2</i>	Ss <i>Kiss1r</i>
Exon 1	261 bp	252 bp
Intron I	2.5 kb	2.5 kb
Exon 2	129 bp	129 bp
Intron II	5.3 kb	0.2 kb
Exon 3	136 bp	131 bp
Intron III	0.9 kb	0.8 kb
Exon 4	239 bp	244 bp
Intron IV	0.4 kb	1.3 kb
Exon 5	381 bp	381 bp

Figure Legends

Figure 1. Nucleotide and deduced amino acid sequence of the Hh *Kiss1r-2*. Numbers indicate the nucleotide position starting at the A of the initial ATG codon (top line, bold) and the amino acid positions starting at the initial methionine (M, bottom line). The seven transmembrane domains (TMDs) are underlined and numbered. The triangles indicate the location the intron-exon boundaries. An asterisk indicates the termination codon.

Figure 2. Amino acid alignment of Atlantic halibut (Hh *Kiss1r-2*) with selected fish *Kiss1r-2* sequences: zebrafish-A (ZfA, **ABV44612**), Atlantic croaker (Ac, **ABC75101**), cobia (Co, **ABG82165**), grey mullet (Gm, **ABG76790**), Nile tilapia (Nt, **BAD34454**) and Senegalese sole (Ss, **EU136710**). Asterisks indicate a perfect match among all aligned sequences whereas dots represent conservative replacements: (.) indicates conserved, and (:) highly conserved. Numbers at the end of each line refer to the amino acid position. The signal peptide and transmembrane domains (TMDs) are boxed. The putative *N*-glycosylation sites are underlined, serine kinase filled by black arrowheads, theonine sites filled by black diamond and tyrosine kinase, filled by black circles. Numbers indicate amino acid number.

Figure 3. Synteny of *Kiss1r-2* in teleost fish. Genomic organization of the zebrafish, pufferfish, fugu, medaka and three-spined stickleback *Kiss1r-2* are from Ensembl (chromosomes 2, 15, scaffold_80, 17 and group III, respectively). The Atlantic halibut sequence is from a 92 kb BAC clone that contains only partial *atp6v0b* and *odf2L* sequences. Abbreviations: b4galt2, β -1,4-galactosyltransferase 2; atp6v0b, V-type proton ATPase 21 kDa proteolipid subunit; hyp. prot., conserved hypothetical protein; miRNA, three predicted

miRNA (miR-23a, miR-27a, miR-24); b0s7g2, novel kinesin-like protein; atp5L, ATP synthase subunit g; rabL5, rab-like protein 5; odf2L, sperm tail outer dense fiber protein 2-like.

Figure 4. Phylogenetic and evolutionary relationships of vertebrate *Kiss1r* sequences. (A) Unrooted phylogenetic tree constructed using the neighbour-joining method of Mega version 4 (Tamura et al., 2007). Sequences were as follows: dog (XM 850105), sheep (ABY61032), rhesus macaque (XP 001117198), mouse (NP 444474), rat (AAD19664), pig (ABE73453), human (AAK83235), chimpanzee (XR 020820), opossum (XM 001374715), platypus (platypus-1, Ultra 266:390150;395924; platypus-1B, Contig 15629:1100:6789), clawed frog (clawed frog-1A, EU85678; clawed frog-1B, EU853679; clawed frog-2, EU853680), medaka (medaka-1, Chromosome 9:4480521:4500733; medaka-2, Chromosome 17:29839761:29855706); zebrafish (zebrafish_A, ABV44612; zebrafish_B, ABV44613); goldfish (goldfish-A, ACK77792; goldfish-B, ACK77793); bullfrog (ACD44939), fathead minnow (ABV45419), Atlantic halibut (GO330487), Nile tilapia (BAD34454), Senegalese sole (ABW96362), grey mullet (ABG76790), cobia (ABG82165) and Atlantic croaker (ABC75101). Three-spined stickleback, fugu, pufferfish, lesser hedgehog, Anole lizard, pika, guinea pig, bushbaby, dolphin, megabat, microbat, kangaroo rat, orangutan, gorilla and mouse lemur were obtained from public databases (Ensembl). (B) *Kiss1r* evolution in vertebrates as deduced from the information available so far. D = duplication.

Figure 5. Conserved upstream regions in fish *Kiss1r-2*. The positions of six conserved regions detected by Meme are indicated relative to the ATG start codon of *Kiss1r-2*. Black boxes correspond to sequences similar to [CT]GTTGCTAGGAGACCC, which has similarity

to the MIF-1 binding site; diagonally striped boxes correspond to [CT]TCCTC[TG]CTCTGCCC; checkered boxes correspond to A[AG][GA][AG]AGCTG[CT][AG][GA][GT]TG; horizontally striped boxes correspond to T[GT]TGTCTCTTT; grey boxes correspond to AGG[GT]CA[CG]ACA[GC][GA][AC]C[TC]C, which has similarity to the estrogen receptor binding site; white boxes correspond to CAATCAT[GT]C[CT]GTA[GT]CCC[AT]C[GT]TG[CT]A[TA]T[GC]AG.

Figure 6. Essential biometry and gonadal development in Atlantic halibut. Total length (TL) (A) and Weight (B) for four different year old-classes (1, 3, 5 and 7 years old). Data as mean $\pm$ S.E.M (n = 4–8). (C) Microscopic characteristic of the different stages of gonadal maturation in males (a-d) and females (e-h) of Atlantic halibut. Spg = Spermatogonia, Spc = Spermatocyte, Spd = Spermatid, Spz = Spermatozoa. Oo = Oocyte, Oc = Ovarian cavity.

Figure 7. Tissue distribution of Hh *Kiss1r-2* at two developmental stages, early puberty (three year old) and mature fish (seven year old). Agarose gels show RT-PCR products obtained from, brain (BR), ovary (OV), testis (TS), liver (LV), muscle (MS), stomach (ST), heart (HT), intestine (IT) and kidney (KD). *β -actin* was included for verifying the presence of mRNA in each sample. No-template control (NTC) was used as a negative control.

Figure 8. Hh *Kiss1r-2* expression in (A) brain and (B) gonads from different year-class fish as determined by qPCR. Results were normalized against *β -actin* RNA levels and corrected for amplification efficiency. The expression level in brain of 1 year-old fish was arbitrarily set at 1. Results in B are expressed as fold-change relative to the reference expression level in

panel A in order to compare relative expression between the two tissues. Data are means $\pm$ S.E.M (n = 4-7). N.d, not detected.

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Figure 1. Mechaly, et al., 2009

	Signal peptide	TMD1	
Zfa	MFSGED-WNSSE--LLNGS-FRNSSME-----DSEGE-HPFLTDAWL	LVPLFFSLIM	47
Ac	MYSTKELWNSTEQIWFNGS-EANLSLGRRGND---EEEEGQHPFLTDAWL	LVPLFFALIM	56
Co	MYSTKELWNSTEQVWINGS-GTNFSLGRRGND---EEEEGQHPFLTDAWL	LVPLFFSLIM	56
Gm	MHSSEEPWNSSEHVWVNGS-EANFSLGRRRVDEEKEEGGQHPFLTDAWL	LVPLFFSLIM	59
Nt	MYSSEELWNSTEQVWINGS-GTNFSLGRHEDD---EEEGDKHPFFTDAWL	LVPLFFSLIM	56
Ss	MYSSKEPWNSTDRVWINGS-RVNVSLERHGDS--EEDEGDQHPFLTDAWL	LVPLFFSLIM	57
Hh	MFSSEEPWNSTEQVWLNSEANFSLGRRGSDDEEEEDGDQHPFLTDAWL	LVPLFFSLIM	60
	. :: ***: .*** * *: : : * ***:*****:***		
	TMD2		
Zfa	LVGLIGNSLVIVISKHRQMRATNFYIANLAATDIIIFLLCCVPFTATLYPLPGWIFGDF		107
Ac	LVGLVGNRSVIVISKHRQMRATNFYIANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		116
Co	LVGLVGNRSVIVISKHRQMRATNFYIANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		116
Gm	LVGLVGNSLVIVISKHRQMRATNFYIANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		119
Nt	LVGLVGNSLVIVISKHRQMRATNFYIANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		116
Ss	LVGLVGNSLVIVISKHRQMRATNFYIANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		117
Hh	LVGVVGNSLVIVISKHRQMRATNFYIAANLAATDIIIFLVCCVPFTATLYPLPGWIFGNF		120
	: *****:*****:*****:***		
	TMD3	TMD4	
Zfa	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPRVAMIVSICIWIGSFIL		167
Ac	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPRVAMIVSICIWIGSFIL		176
Co	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPRVAMIVSICIWIGSFIL		176
Gm	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPKVAMIVSICIWIGSFIL		179
Nt	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPKVAMIVSICIWIGSFVL		176
Ss	MCKEFAFLQQVTVQATCITLTAMSGDRCYVTVYPLKSLRHRTPRVAMIVSLCIWIGSFIL		177
Hh	MCKEFAFLQQVTVQATCITLTAMSGDRCYITIYPLKSLRHRTPRVAMIVSICIWIGSFIL		180
	*****:*****:*****:*****:*****:*****:***		
	TMD5		
Zfa	STPIFLYQRIEDGYWYGPRKYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLK		227
Ac	STPILVYQRIEDGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLVK		236
Co	STPILMYQRIEGGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLVK		236
Gm	STPILMYQRIEGGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTITFCYTFMLVK		239
Nt	STPILMYQRIEGGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLVK		236
Ss	STPILMYQRIEDGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLVK		237
Hh	STPILMYQRIEGGYWYGPRQYCMERFPSKTHEKAFILYQFIAAYLLPVLTISFCYTFMLVK		240
	* **::**:*:*****:*****:*****:*****:*****:***:***:***		
	TMD6		
Zfa	RVGQASVEPVDNNHGVHLLSERTISIRSKISKMVVVIVVLFITICWGPIQIFVLFQSFYPN		287
Ac	RVGQPTVEPVDNNYQVNLLSERTISIRSKVPMVVVIVVLLFAICWGPIQIFALFQSFYPN		296
Co	RVGQPTVEPVDNNYQVNLLSERTISIRSKVSKMVVVIVVLLFAVCWGPIQIFALFQSFYPN		296
Gm	RVGQPTVEPVDNNYQVNLLSERTISIRSKVSKMVVVIVVLLFAVCWGPIQIFVLFQSFYPN		299
Nt	RVGQPTVEPVDNNYQVNLLSERTISIRSKVSKMVVVIVVLLFAICWGPIQIFVLFQSFYPN		296
Ss	RVGQPTVEPVDNNYQVNLLSERTISIRSKVSKMVVVIVVLLFICWGPIQIFVLFQSFHPN		297
Hh	RVGQPTVEPVDNNYQVNLLSERTLSIRSKVSRMVVVIVVLLFAICWGPIQIFVLFQSFHPN		300
	****:*****:***:*****:*****:*****:*****:***:*****:***		
	TMD7		
Zfa	FKANYATYKIKITWANCMSYANSSINPIVYGFMGASFQKSFRTFPFLFKHKVRDSSVASR		347
Ac	YQHNYATYKIKITWANCMSYANSSVNPIVYGFMGATFQKSFRTFPFLFKHKVRDSSMASR		356
Co	YQHNYATYKIKITWANCMSYANSSVNPIVYGFMGASFQKSFRTFPFLFKHKVRDSSMASR		356
Gm	YQPNYATYKIKITWANCMSYANSSVNPIVYGFMGASFQKSFRTFPFLFKHKVRDSSMASR		359
Nt	YQPNYATYKIKITWANCMSYANSSVNPIVYGFMGASFQKSFRTFPFLFKHKVRDSSMASR		356
Ss	YRPNYVTYKIKITWANCMSYANSSVNPIVYGFMGASFQKSFRTFPFLFKHKVRDSSMASR		357
Hh	YRPNYVTYKIKITWANCMSYANSSVNPIVYGFMGASFQKSFRTFPFLFKHKVRDSSMASR		360
	: : **.******:*****:*****:*****:*****:***		
Zfa	TANAEIKL-----		355
Ac	TANAEIKFVAEEGNNNNAMN		377
Co	TANAEIKFVAEEGNNNNAMN		377
Gm	TANAEIKFVAEEGNNNNAVN		380
Nt	TANAEIKFVAEEGNNNNAVN		377
Ss	TANAEIKFVAEEGNNNNAMN		378
Hh	TGNAEIKFVAEEGNNNNAVN		381
	*.*****:		

Figure 2. Mechaly, et al., 2009

Figure 3. Mechaly, et al., 2009

Figure 4. Mechaly, et al., 2009

Figure 5. Mechaly, et al., 2009

Figure 6. Mechaly, et al., 2009

Figure 7. Mechaly, et al., 2009

Figure 8. Mechaly, et al., 2009